Molecular aspects of ageing and the onset of leaf senescence

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Chapter **4**

A role for cytokinin in the onset of leaf senescence by ethylene in *Arabidopsis*

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Abstract

The expression of several *Arabidopsis* senescence-associated genes (SAGs) was found to be upregulated during leaf development of the *onset of leaf death 9* mutant under standard growth conditions. Treatment of the mutant with ethylene resulted in a severe senescence response both phenotypically as well as on transcript level. A RNAi-based strategy to downregulate the expression of a SAG encoding for a hydroxyproline-rich glycoprotein, attenuated the ethylene induced senescence response of *old9*. Comparison of the *old9* transcriptome revealed that 67% of the differentially expressed genes with wild type are cytokinin responsive. Cytokinins are well known for their effect to retard leaf senescence. However, by applying cytokinin early in development the response to ethylene-induced senescence could be enhanced. We argue that the cytokinin effect we observed is the result of increasing the sink strength of young tissue while opposite to that ethylene promotes source strength of old leaves. Therefore we propose that the balance between sink and source tissue can modulate the progression rate and timing of senescence.

Introduction

The development of a leaf starts with the formation of a leaf primordium from the shoot apical meristem (Van Lijsenbettens and Clarke, 1998). A subsequent differentiation and expansion of the different cells gives rise to a mature leaf consisting of an epidermis, a mesophyll layer and vascular tissue (Pyke et al., 1991). The determinate program of leaf development ends with the age dependent onset of senescence resulting in the death of the tissue. However, this final stage of leaf development is critical for plant fitness as nutrient relocation to young developing parts is achieved through this process. Leaf senescence is thus an evolutionary selected developmental process and represents an important phase in the plant life

cycle (Bleecker, 1998; Buchanon-Wollaston et al., 2003). The study of leaf senescence will not only increase our understanding of a fundamental biological process, but also contributes to means to control leaf senescence to improve traits of crops and ornamental plants including postharvest storage.

The development of a leaf is as a mitotic event which occurs prior to unfolding and cell expansion is initiated. The mitotically active cells that form the leaf primordium arise from the shoot apical meristem (SAM), which contains a pluripotent stem cell population (Bowman and Eshed, 2000). The initiation of a leaf from this population of stem cells involves the action of several phytohormones. First the maintenance of the stem cells requires biosynthesis of cytokinin (Bowman and Eshed, 2000). However, the initiation of a leaf primordium requires gibberellin (GA), which accumulates due to the repression of cytokinin biosynthesis (Yanai et al., 2005; Jasinski et al., 2005). Plants with reduced cytokinin production have less leaves and contain fewer leaf cells (Werner et al., 2001) which was interestingly also shown in the triple mutant for a cytokinin receptor *arabidopsis histidine kinase2* (*ahk2*), *ahk3*, *ahk4* (Nishimura et al., 2004). On the other hand transgenic *Arabidopsis* plants which overproduce cytokinin have more leaf cells (Rupp et al., 1999). Thus cytokinin has a regulatory function in leaf cell formation. The cell division in the leaf primordium of *Arabidopsis* sets an age-gradient from “old” cells at the leaf tip and margins to younger cells at the base of the leaf (Ferreira et al., 1994).

The post mitotic phase of leaf development involves cell expansion until the final size is reached at maturity. The expansion is controlled in part by the *ANGUSTIFOLIA* gene in *Arabidopsis* which acts through arrangement of cortical microtubules and the expression of cell-wall loosening genes (Kim et al., 2002). During the expansion of mesophyll cells, partially differentiated chloroplasts start to divide actively (Pyke and

Leech, 1994) to attain photosynthetic competence of a leaf. *Arabidopsis* grown in medium containing cytokinin shows an accelerated photomorphogenesis phenotype coinciding with induced expression of photosynthetic genes (Schmülling et al., 1997; Brenner et al., 2005). Several effects of cytokinin can also be induced by light, including leaf expansion, chlorophyll accumulation and inhibition of hypocotyl elongation. Although both cytokinin and light can induce similar processes the action of light involves a phytochrome-dependent pathway while cytokinin in part requires the ethylene response pathway (Cary et al., 1995; Su and Howell, 1995). Interestingly, cytokinin induces the expression of nitrate reductase in plants (Samuelson et al., 1995; Taniguchi et al., 1998) and was suggested to have a regulatory role in nitrate assimilation (Tischner, 2000). Cytokinin affects source-sink relationships and causes nutrient accumulation in leaves treated with the phytohormone (Roitsch and Ehness, 2000). The transport of carbon and nitrogen compounds from source leaves to sink leaves requires glucose, sucrose, and amino acid transporters which have been identified in the *Arabidopsis* genome as large gene families (Lalonde et al., 2004). One of these sucrose transporters AtSUC2 was shown to be developmentally regulated and is induced during the sink-to-source transition of leaves (Truernit and Sauer, 1995). The application of cytokinin to seedlings results in a downregulation of *AtSUC1* (Brenner et al, 2005) while overexpression of *ARR22* (Kiba et al., 2004) results in down regulation of both *AtSUC1* and *AtSUC2* suggesting that cytokinin can modulate the transition of source-to-sink. The final stage of leaf development results in a total commitment to source function and results in massive nutrient remobilisation by the onset of leaf senescence (Balibrea Lara et al., 2004). In *Arabidopsis* the onset of leaf senescence occurs in a leaf-age dependent manner (Gan and Amasino, 1997; Quirino et al.,

2000). Ageing of the leaves starts with the initiation of a leaf primordium and occurs throughout the development resulting in senescence and death (Lim et al., 2003). The developmental changes during the lifespan of a leaf can be viewed as age-related changes (ARCs) that set the developmental age and thus determine if a leaf can undergo senescence (Jing et al., 2005; Schippers et al., 2007). The onset of senescence can be delayed by the application of cytokinin on leaf senescence (Richmond and Lang, 1957) which is thus acting as an anti-ageing factor. In addition, expression of cytokinin biosynthesis genes under the promoter of a senescence-associated gene (SAG) delays the initiation of leaf senescence in several plant species (Gan and Amasino, 1995; McCabe et al., 2001; Khodakovskaya et al., 2005). The SAM gene *knotted1* (*kn1*) regulates cytokinin content and delays leaf senescence when expressed under the control of a SAG promoter in tobacco (Ori et al., 1999). The mechanism of cytokinin-mediated delay of senescence is dependent on an extracellular invertase. Activation of the invertase increases the sink strength of the tissue and the rate of sugar utilization which results in delayed onset of senescence (Balibrea Lara et al., 2004). These studies suggest that sugar metabolism mediated by cytokinin is an important factor in determining the onset of leaf senescence and that the transition of sink-to-source is an important ARC during the development of a leaf.

In our attempt to elucidate factors that control the onset of leaf senescence we make use of the senescence inducing phytohormone ethylene. In the present study, we selected the *Arabidopsis onset of leaf death 9* (*old9*) mutant. The mutant was shown in chapter 2 to senescence early upon ethylene treatment. Since the effect of ethylene is age-dependent the mutant presents a good starting point for determining regulation of age-related changes (ARCs). Here we present evidence for a role of

cytokinin on the timing of ethylene-induced senescence in *Arabidopsis*. The mutant *old9* shows altered expression of cytokinin responsive genes. Furthermore we demonstrate that exogenous application of cytokinin can prime a leaf for ethylene-induced senescence and argue it to be caused by changes in the sink/source strength of the leaves.

Results

old9 presents a co-dominant locus involved in ethylene-inducible senescence

The *old9* mutant of *Arabidopsis* was isolated initially as an early leaf senescence mutant upon ethylene treatment (Jing et al., 2005). *old9* is classified as a class II mutant (Jing et al., 2002) and was found to develop as wild type under standard growth conditions. Since the induction of leaf senescence by ethylene is dependent on the age of the leaf (Grbić and Bleecker, 1995, Jing et al., 2002) we anticipate that *old9* might be involved in the integration of ARCs and environmental stimuli to regulate the onset of senescence.

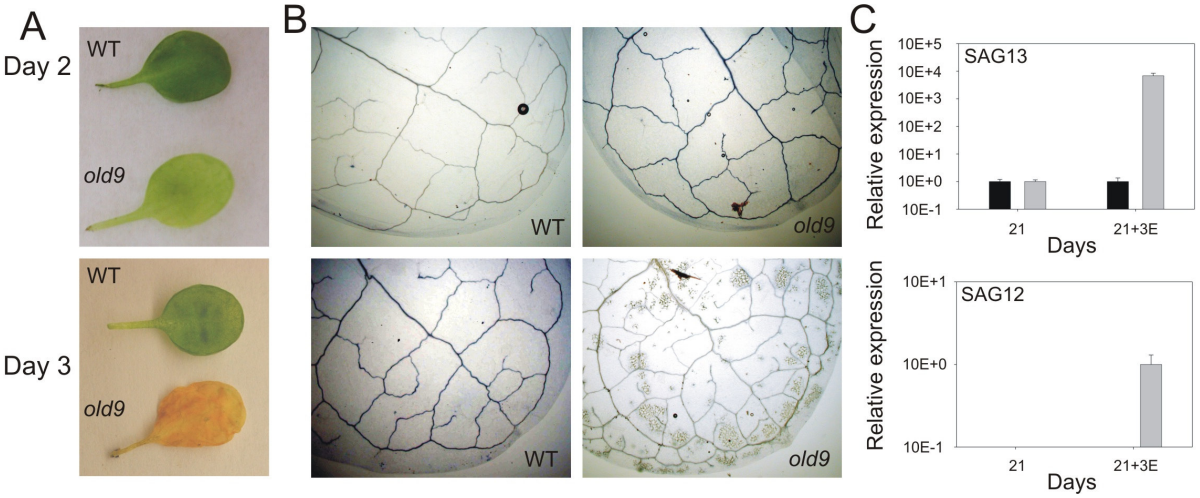

Figure 1. Senescence response of *old9* and wild type leaves to ethylene treatment. Wild type and *old9* plants were grown for 21 days in air and were subsequently treated for 2 or 3 days with ethylene. Representative leaves from the first true leaf pair were photographed (A) or stained with tryphan blue (B) as described in the Methods section. (C). Transcript accumulation of the senescence-associated genes *SAG12* and *SAG13* after ethylene treatment in *old9* (gray bars) and wild type (black bars). The values shown are the means of three repeats ± SD (indicated by error bars).

For the characterization of the mutant we first examined the senescence symptoms during ethylene-induced senescence. The first leaf pair of *old9* has reduced leaf longevity upon ethylene treatment at 21 days as observed visually (Figure 1A). After 2 days the leaves become pale green and after 3 days the entire leaf turned yellow which indicated the complete execution of senescence (Grbić and Bleecker, 1995). In contrast, leaves of the wild type show visual senescence symptoms after 4 days of ethylene treatment (data not shown). After 3 days of treatment cell death is observed in the mutant but not in the wild type (Figure 1B). The appearance of cell death occurs in an ordered way starting from the leaf tip. The onset of leaf senescence is marked by increased expression of senescence associated genes (SAGs) (Nam, 1997). Expression of the *SAG12* and *SAG13* genes increased dramatically after 3 days of ethylene treatment in *old9* mutants (Figure 1C). Taken together, the complete yellowing of the leaf, appearance of cell death and SAG gene expression upon ethylene treatment are consistent with the suggested occurrence of early ARCs in the mutant (Jing et al., 2005).

A backcross of the *old9* mutant revealed that it segregates as a co-dominant trait both in the original mutant accession *Landsberg* (L*er*) and the mapping background Columbia (Col). The position of the *old9* locus was narrowed down to a region on chromosome II of 146kb on BAC F6E13 and F4I1. The identified region contains 54 annotated genes which are summarized in Table 1. In an attempt to identify the mutation seven genes have been sequenced but were shown to be identical to the wild type gene.

Table 1. List of genes in mapped *old9* area. Shown is the data from the TAIR annotation and a Pubmed record when available. Asterisks indicate genes that were sequenced and found to be identical to the wild type.

TAIR- ID	Name	Description	PubMed
at2g43960		similar to SWAP	NF
at2g43970		La domain-containing protein	NF
at2g43980	ITPK4	inositol 1,3,4,5-tetrakisphosphate isomerase	17698066
at2g43990		Unknown protein	NF
at2g44000		Unknown protein	NF
at2g44010		Unknown protein	NF
at2g44020		mitochondrial transcription termination factor-related	NF
at2g44030		Kelch-repeat containing F box	NF
at2g44040		dihydrodipicolinate reductase; lysine biosynthesis	15652176
at2g44050	COS1	6,7-dimethyl-8-ribityllumazine (DMRL) synthase	10419541
at2g44060		late embryogenesis abundant family protein	NF

at2g44065		Ribosomal protein L2	NF
at2g44070		eukaryotic translation initiation factor 2B family protein	NF
at2g44080*	ARL	AGROS-like gene involved in cell expansion; BR induced	16824178
at2g44090		Unknown protein	NF
at2g44100*	ATGDI1	Guanoside diphosphate dissociation inhibitor	8953772
at2g44110*	MLO15	7 transmembrane (7TM) G-protein-coupled receptor	16525893
at2g44120	RPL7C	60S ribosomal protein L7	15821981
at2g44130*		Kelch-repeat containing F box	NF
at2g44140*	ATG4a	Autophagy 4a	15178341
at2g44150*	ASHH3	Histone-Lysine N methyl transferase	17295027
at2g44160	MTHFR2	methylenetetrahydrofolate reductase	NF
at2g44170	NMT2	N-myristoyltransferase	12912986
at2g44175		Unknown protein	NF
at2g44180	MAP2a	Methionine Aminopeptidase	11060042
at2g44190		Unknown protein	NF
at2g44195		Unknown protein	NF
at2g44200		Unknown protein	NF
at2g44210		Unknown protein	NF
at2g44220		Unknown protein	NF
at2g44230		Unknown protein	NF
at2g44240		Unknown protein	NF
at2g44250		Unknown protein	NF
at2g44260		Unknown protein	NF
at2g44270		Unknown protein	NF
at2g44280		similar to lactose permease-related	NF
at2g44290		protease inhibitor/seed storage/lipid transfer protein	NF
at2g44300		lipid transfer protein-related	12805588
at2g44310		calcium-binding EF hand family protein	NF
at2g44320*		TRNA serine	NF
at2g44330		zinc finger (C3HC4-type RING finger)	15644464
at2g44340		VQ motif-containing protein	NF
at2g44350	ATCS	Citrate synthase 4	8979399
at2g44360		Unknown protein	NF
at2g44370		DC1 domain-containing protein	NF
at2g44380		DC1 domain-containing protein	NF
at2g44390		DC1 domain-containing protein	NF
at2g44400		DC1 domain-containing protein	NF
at2g44410		protein binding / zinc ion binding	NF
at2g44420		protein N-terminal asparagine amidohydrolase family protein	NF
at2g44430		DNA-binding bromodomain-containing protein	NF
at2g44440		emsy N terminus domain-containing protein	NF
at2g44450		glycosyl hydrolase family 1 protein	16287169
at2g44460		putative thioglucosidase gene	17063378
at2g44470		glycosyl hydrolase family 1 protein	NF
at2g44480		glycosyl hydrolase family 1 protein	NF

Transcriptional analysis of air-grown old9

As mentioned before the induction of senescence by ethylene is age-dependent (Hensel et al., 1993; Jing et al., 2002) in Arabidopsis. Therefore *old9* might represent a locus that monitors the age of a leaf to set the developmental program accordingly. Since the mutant shows normal sensitivity to ACC (see chapter 2), we suggest that

an early age-related change (ARC) occurs in the mutant priming the leaves for senescence. Micro-array analysis was used to identify differentially expressed transcripts which could function as a marker for possible ARCs in the mutant. Transcript levels were assessed in the first leaf pair of *old9* and wild type after 21 days of growth under standard conditions thus when no visual difference between the two genotypes is observed. 89 genes were found to be differentially regulated in the combined biological and technical replicates of air-grown plants (Table 2).

Table 2. Genes differentially expressed in *old9* after 21 days of growth under standard conditions. The 89 genes listed show statistical significance versus the wild type control ($P < 0.05$, Student's t test). Shown are TAIR ID, gene name, description, Pubmed ID and ratio. BA indicates ratio found during micro-array study on genes responsive to cytokinin (Brenner et al., 2005). (S). Senescence associated (-, no; +, yes)

TAIR- ID	Name	Description	PubMed	Ratio	BA	S
At1g01090	PDH-E1	pyruvate dehydrogenase alpha;	9393637	1.61	1.51	-
At1g04820	TUA4	Tubulin alphaL-4 chain; cytoskeleton	1498608	0.57	0.71	-
At1g08200	AXS2	UDP-D-apiose/UPD-D-xylose synthetase	12969423	0.29	ND	-
At1g11840	AtGLX1	lactoylglutathione lyase activity	NF	0.30	0.50	-
At1g12840	DET3	V-ATPase; cell expansion and photomorphogenesis	11604454	0.41	1.15	+
At1g16850		Role in NaCl and cold stress; anthocyanin synthesis	17888165	0.47	0.22	-
At1g20510	OPCL1	Jasmonic acid biosynthesis; wound-induced	16963437	1.66	0.38	+
At1g21312		RNA recognition motif (RRM)-containing protein	NF	3.58	1.21	-
At1g21520		Unknown protein	NF	0.46	1.50	+
At1g23040		hydroxyproline-rich glycoprotein	12805588	3.01	0.5	+
At1g28380	NSL1	Necrotic spotted lesions; plant defense	16900325	1.42	0.61	-
At1g32470		glycine cleavage system	14671022	0.61	1.23	-
At1g35710		leucine-rich repeat transmembrane protein kinase	NF	2.61	1.03	-
At1g37130	NR2	Nitrate reductase	3393528	3.52	1.94	-
At1g67105		Senescence-associated Gene	NF	19.56	ND	+
At1g67740	PSBY	PsbY precursor Part of Photosystem II	9829828	1.72	1.48	+
At1g67865		Unknown protein	NF	1.65	1.19	+
At1g68190		zinc finger (B-box type) family protein	NF	1.57	1.74	-
At1g69935		Unknown protein	NF	0.61	1.13	-
At1g71030		MYB transcription factor repressed by BZR1	15681342	1.36	5.27	-
At1g76990	ACR3	amino acid-binding protein	12481063	1.53	1.77	-
At1g78710		Stress related; unknown protein	NF	0.31	0.56	-
At1g78995		Unknown protein	NF	0.65	0.09	-
At1g55490	CPN60B	beta subunit of the chloroplast chaperonin 60	12668771	0.59	0.59	-
At2g06850	EXGT-1A	Endo-xyloglucan transferase; br responsive	11673616	0.60	1.08	-
At2g18700	TPS11	Trehalose biosynthesis	11520870	1.62	8.17	+
At2g19620		Ndr family protein; cell differentiation	17565583	0.45	0.68	-
At2g20120	COV1	Vascular patterning	12668628	1.54	1.58	-
At2g21130	CYP1	Cyclophyllin	17473866	0.54	0.97	-
At2g24850	TAT3	Tyr amino transferase	9342878	6.94	1.73	+
At2g25490	EBF1	F-box binds EIN3; OE ethylene insensitive	14675533	1.93	1.73	-
At2g25950		Unknown protein	NF	0.32	0.69	-
At2g26560	PLP2	Phospholipase induced by pathogen attack	16297072	1.93	0.48	+
At2g29630		thiamine biosynthesis family protein	NF	1.66	1.27	-
At2g30230		Unknown protein	NF	0.31	ND	+

At2g31890		Unknown protein; endomembrane system	NF	1.86	0.82	-
At2g32670	VAMP275	Vesicle associated membrane protein	14500793	3.24	1.67	-
At2g40000		Putative nematode resistance gene	15448178	1.57	4.31	+
At2g40080	ELF4	Photoperiodism; red light response; PhyB signaling	14605220	0.59	0.13	+
At2g40360		putative WD-40 repeat protein; BOP1 domain	NF	0.19	0.61	-
At2g43100		aconitase C-terminal domain-containing protein	NF	2.18	0.08	-
At2g46450	CNGC12	Cyclic nucleotide gated channel; fungus response	16461580	3.61	0.78	-
At2g47960		Transglutaminase, putative	NF	1.61	1.74	-
At2g29080	FTSH3	Ftsh protease; repair PSII	14630971	0.65	0.89	-
At3g01490		Putative MAPKKK, MRK1-like; sucrose phospho	NF	0.64	ND	-
At3g06710		similar to protein binding / zinc ion binding	NF	2.08	1.24	-
At3g10020		similar to Os12g0147200	NF	1.51	4.18	-
At3g10260		reticulon family protein	NF	0.08	0.65	-
At3g11410	AtPP2CA	Protein phosphatase 2C, represses ABA	16361522	1.66	0.58	+
At3g16520		UDP-glucoronosyl/UDP-glucosyl transferase protein	NF	1.99	1.21	-
At3g18490		aspartyl protease family protein	NF	1.85	0.78	-
At3g20790		oxidoreductase family protein	NF	0.14	0.54	-
At3g22320	RPABC24	RNA polymerase I, II and III 24.3 kDa subunit	NF	0.62	1.25	-
At3g23700		S1 RNA-binding domain-containing protein	NF	1.35	1.05	-
At3g23820	GAE6	UDP-D-glucuronate 4-epimerase; pectin synthesis	15225656	1.59	0.82	-
At3g48360	BT2	Involved in TAC1-mediated telomerase activation	17220202	0.23	9.87	-
At3g55240		Unknown protein; OE Pseudo Etiolation in Light	17227551	1.53	ND	-
At3g57260	BGL2/PR2	beta 1,3-glucanase	1824335	5.48	0.31	+
At4g12830		hydrolase, alpha/beta fold family protein	NF	0.13	0.60	-
At4g14890		ferredoxin family protein; light protection	NF	0.65	1.46	-
At4g17390		60S ribosomal protein L15	NF	0.66	1.20	-
At4g18200	AtPUP7	Putative cytokinin and purine transporter	10662864	1.61	nd	-
At4g19170	NCED4	Nine-cis-epoxycarotenoid dioxygenase 4	15862093	2.02	1.88	-
At4g19860		lecithin:cholesterol acyltransferase	NF	1.44	1.33	+
At4g28220	NDB1	NAD(P)H DEHYDROGENASE B1	17673460	1.87	1.23	-
At4g32020		Unknown XRN4 target	NF	1.70	0.45	+
At4g34740	ATASE2	de novo purine biosynthesis	15266056	0.60	0.88	-
At4g35090	CAT2	peroxisomal catalase; photosynthetic active tissue	17080932	1.46	1.47	+
At4g36020	CSDP1	Encodes a cold shock domain protein	17169986	0.63	1.44	-
At4g36040		DNAJ heat shock like protein	NF	1.92	4.73	+
At5g02500	HSC70	Heat shock cognate 70 kDa protein	12805626	4.61	1.82	-
At5g03850		40S ribosomal protein S28	NF	0.62	1.37	-
At5g08050		Thylakoid protein	NF	0.63	1.30	-
At5g08520		MYB family transcription factor	NF	1.37	1.03	-
At5g10760		aspartyl protease family protein	NF	2.07	0.91	-
At5g15650	RGP2	alpha-1,4-glucan-protein synthase	9536051	0.49	ND	-
At5g16150	GLT1	plastidic glucose exporter, trehalose responsive	7031512	1.51	1.51	+
At5g17460		Unknown mitochondrial protein	NF	0.53	0.60	-
At5g18630		triacylglycerol lipase	NF	1.60	1.32	+
At5g19140		auxin-responsive genes	NF	1.84	1.20	+
At5g22580		Putative monooxygenase	15213437	0.43	1.13	-
At5g24530		flavanone 3-hydroxylase like protein	NF	2.43	1.27	-
At5g35180		EDR2-like (enhanced disease resistance 2)	NF	1.71	0.63	-
At5g37475		translation initiation factor-related	NF	0.30	ND	-
At5g40450		Unknown protein	NF	4.60	0.62	+
At5g43060		RD21-like cysteine proteinase, sugar responsive	NF	1.36	1.20	-
At5g47560	ATTDT	malate transmembrane transporter	12947042	2.12	3.46	+
At5g55960		Unknown protein	NF	0.66	0.81	-
At5g57760		DNA binding protein, MPK4 dependent	16813576	0.24	3.68	-

To confirm the microarray data we performed real-time PCR analysis of several identified genes as shown in Figure 2. NIA2, TAT3 and EBF1 were confirmed to be up regulated in air-grown *old9* compared to the wild type. Moreover the cell wall D-apiose synthetase (AXS2) is downregulated according to the RT-PCR analysis confirming the microarray data. Thus the RT-PCR analysis validated the genes identified by the microarray experiment.

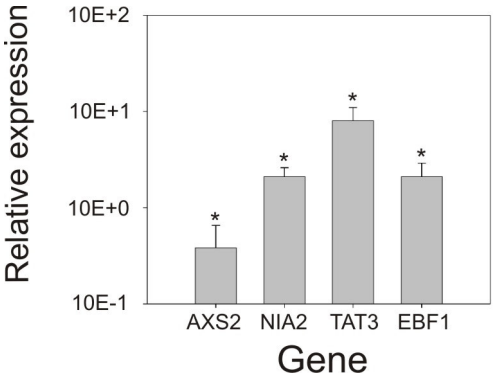

Figure 2. Real-Time PCR validation of transcripts identified by microarray analysis *of old9*. Transcript levels are shown as ratio between wild type and mutant. The values shown are the means of three repeats ± SD (indicated by error bars). Experiments were repeated once and similar results were obtained. Asterisks indicate statistical significance versus the wild type control in each case (* $P < 0.05$, Student's t test).

Microarray analysis results in large datasets of differentially expressed genes, however, these datasets are insignificant without a biological-orientated analysis. Therefore the identified genes that are differentially expressed in the mutant were studied by their annotation and classification in the TAIR database and by literature search. The developmental program of young leaves is regulated and controlled by many genes, interestingly several early leaf developmental genes are differentially expressed in *old9* plants as compared to the wild type. DET3 (At1g12840), encodes for a subunit of a vacuolar ATPase involved in cell elongation during leaf development (Schumacher et al., 1999). The expression of DET3 decreases during the development of the leaf, and the mutant has a more than 2-fold decreased expression compared to the wild type. Formation of vascular tissue is repressed by COV1 (At2g20120) which when mutated results in increased vascular tissue in *Arabidopsis* (Parker et al., 1999). In *old9* an increased expression of this repressor is detected at 21 days. Telomerase activity is shut down after mitotic division of cells by decreasing the expression of its activator BT2 (At3g48360) (Ren et al., 2007), BT2 is 4 fold downregulated in *old9* compared to wild type. The altered expression of these

transcripts in *old9* is consistent with the suggestion that certain ARCs occur earlier in the mutant.

Next to that transcripts were identified that are involved in genetic pathways implicated in the biosynthesis or response to phytohormones. Ethylene promotes the onset of leaf senescence in many plant species and in *old9* an increased expression of the negative regulator of ethylene responses EBF1 (At2g25490) (Potuschak et al., 2003) was observed. Besides that OPCL1 (At1g20510), involved in the biosynthesis of Jasmonic acid (Koo et al., 2006) shows increased expression in *old9*. Furthermore a negative regulator of ABA signaling, PP2CA (At3g11410), is more abundantly expressed in the mutant (Kuhn et al., 2006). Thus, the *old9* mutation may cause a partial repression of ABA and ethylene signaling after 21 days of growth in air. Noteworthy is the observation that several genes encoding for cell-wall modifying enzymes are differentially expressed in *old9*. Two genes involved in cross-linking of the cell wall are differentially expressed; a D-apiose gene is downregulated (At1g08200) while a hydroxyproline-rich glycoprotein (HRGP) is up regulated (At1g23040). Next to that a xyloglucan transferase (At2g06850) is down regulated while a gene involved in pectin synthesis (At3g23820) is up regulated. Furthermore, an alpha-1,4-glucan synthase (At5g15650) is down regulated while beta-1,3-glucanase is more than 5-fold increased in expression. This suggests that the cell wall of *old9* might have a different composition than the wild type. Interesting to note is the increased expression of a putative cytokinin transporter (At4g18200) and the up regulation of nitrate reductase (At1g37130).

To gain further insight in the air-grown transcriptome of *old9* a comparison with other microarray experiments was carried out by making use of the GENEVESTIGATOR tool (Zimmerman et al., 2004). The analysis revealed that 67% of the differentially expressed genes in *old9* also respond to a 2 hour cytokinin treatment in 8 day old seedlings (Brenner et al., 2005) (Table 2). Since cytokinin prevents senescence this observation might explain the absence of early senescence under normal growth conditions. However, it can not be ruled out that altered expression of cytokinin responsive genes might cause the early onset of senescence by ethylene. Of the 89 transcripts, 24 are senescence associated as shown by Guo et al., (2004). Moreover, At1g67105 is 19.5 fold increased in *old9* and was shown to be upregulated during leaf senescence (data not show). Also TAT3 and BGL2 are induced during leaf senescence and are strongly up regulated in *old9.* These results are consistent with

the notion that *old9* mutants may experience early ARCs and suggests that *old9* leaves may be primed for senescence or that the senescence program may be partially induced during normal growth conditions.

To determine a common regulation between the differentially expressed genes in *old9*, we used coexpression analysis within the ATTED-II database (Obayashi et al., 2007). The coexpression analysis reveals gene-to-gene relationships and is therefore a useful tool to identify gene networks. 29 of the 89 identified genes have an association with one or more other genes. Their association is presented in Figure 3 and shows 3 gene networks containing 3 or more genes and 6 interactions between 2 genes. The largest network contains 8 genes of which 2 are involved in lipid metabolism (AT4g19860 and At5g18630), 3 chloroplastic genes (NCED4, At4g36040 and At5g19140), a gene involved in trehalose metabolism (TPS11) and a MYB transcription factor (At1g71030). The second network is built around the *BGL2* (At3g57260) which encodes a 1,3-beta-glucosidase involved in hydrolyzing fungal cell walls (Mauch et al., 1988) and TAT3 gene that encodes for an enzyme concerned in the synthesis of radical scavengers tocopherols from tyrosine (Hématy et al., 2007). These 2 networks consist for 50 to 66% out of SAG genes which are late leaf developmental genes and thus suggest an advanced leaf developmental program in *old9*. The third network is built around a sucrose-regulated putative MAPKKK (Niittylä et al., 2007).

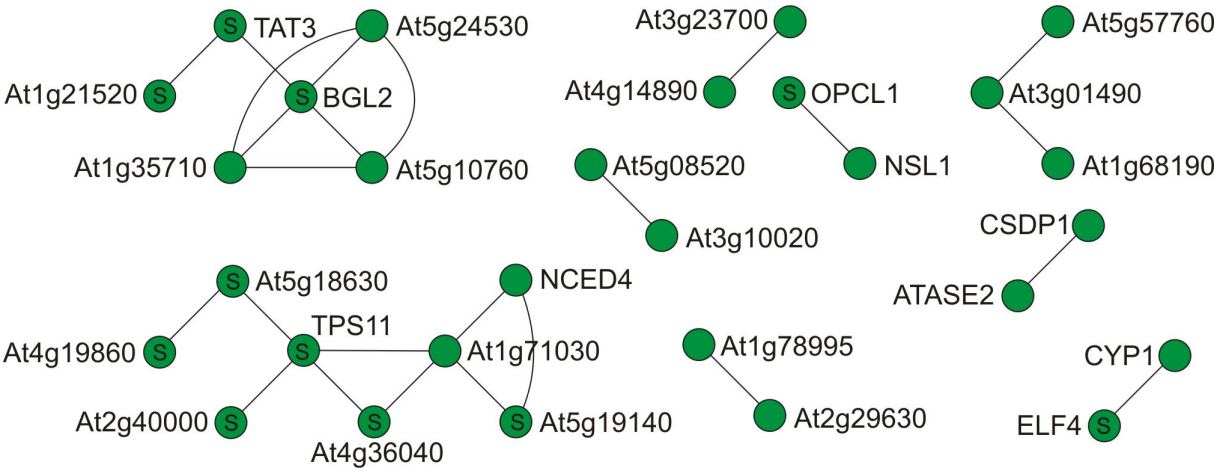

Figure 3. Gene-gene relation between the identified *old9* microarray transcripts was determined by coexpression analysis. Genes are shown as green spheres and a relation between two genes is represented by lines. Lines are only drawn between genes with Pearson correlations >0.6. An S indicates that the gene is senescence associated.

The other 2 genes encode a putative CONSTANS-like transcription factor (At1g68190) and an unknown DNA-binding domain-containing protein (At5g57760). Taken together this analysis suggests that on the transcriptional level the leaf developmental program of *old9* is more progressed. Furthermore it suggests that cytokinin might be an important factor in controlling the *old9* phenotype.

Onset of senescence in old9 after ethylene treatment

The accelerated execution of senescence in *old9* by ethylene was investigated by making use of microarray analysis. We performed the microarray after 8 hours of ethylene treatment when both wild type and mutant show no sign of visible yellowing. We prefer this timepoint because ethylene can induce thousands of transcripts within 24 hours and next to that the progression of senescence involves a massive transcriptional change. We anticipated that by limiting the length of the ethylene exposure only genes involved in the start of leaf senescence and ethylene signaling will show up, facilitating the analysis of the dataset. However, after ethylene treatment hundreds of transcripts were identified that showed altered expression between the mutant and the wild type. To focus our analysis we only investigated genes that are more than 3-fold changed in expression (Table 3).

Table 3. Genes differentially expressed in *old9* after 8 hours of ethylene treatment. 38 transcripts are more than 3-fold induced ($P < 0.05$, Student's t test). Shown are TAIR ID, gene name, description, Pubmed ID and ratio. E indicates ethylene-inducible ; S indicates senescence associated; D indicates defense related. Genes less than 1.5-fold change are considered as not changed represented by 0. +, positive correlation, - negative correlation.

TAIR- ID	Name	Description	PubMed	Ratio	E	S	D
At1g03850		Glutaredoxin family protein, response cytokinin	16212609	3.2	0	+	+
At1g06000		flavonol-7-O-rhamnosyltransferase	17314094	3.1	-	-	-
At1g16260	WAKL8	Wall-associated kinase like 8	12068092	3.9	0	+	0
At1g19380		Unknown protein	NF	3.2	+	-	+
At1g33960	AIG1	Avirulence induced gene	8742710	9.4	0	+	+
At1g56660		Similar to wound-inducible KED of tobacco	10945337	4.3	0	+	0
At1g73650		3-oxo-5-alpha-steroid 4-dehydrogenase like	NF	3.3	0	+	0
At1g74590	ATGSTU10	Glutathione S-transferase	12090627	4.3	0	+	+
At1g75830	PDF1.1	Plant defensin protein	15955924	8.2	+	-	+
At2g29350	SAG13	Senescence associated; alcohol dehydrogenase	10444084	3,5	+	+	+
At2g29720	CTF2B	flavoprotein monooxygenase	NF	4,6	0	-	0
At2g30140		UDP-glucoronosyl/UDP-glucosyl transferase	NF	3,3	+	+	+
At2g32240		similar to kinesin	NF	3,3	+	+	+
At2g32670	VAMP275	Vesicle associated membrane protein	14500793	3,6	0	-	0
At2g43570		Putative endochitinase	15915637	3,2	-	-	+
At2g43680	IQD14	calmodulin binding, calcium signaling	16368012	3,2	0	-	0

At3g13950		Ndr1 dependent	17181774	9,1	-	-	+
At3g18250		Unknown protein	NF	4,8	0	+	+
At3g22060		Unknown protein	17761682	3,2	+	+	+
At3g23550		Multi antimicrobial extrusion protein	NF	3,3	+	-	+
At3g25882	NIMIN-2	a kinase that physically interacts with NPR1/NIM1	15749762	3,7	N	-	N
At3g44700		Unknown protein	NF	132.0	0	-	-
At3g54920	PMR6	Powdery mildew resistant 6; pectate lyase-like	12215508	160.0	0	-	-
At3g62770	ATG18	autophagosome formation	15860012	5,0	0	-	0
At4g14450		C terminal similar to hydroxyporline rich glycoprotein	NF	3,1	N	-	N
At4g17500	ATERF1	Ethylene responsive element binding factor 1	10715325	3,3	+	+	+
At4g25110	ATMC2	Metacaspase 2	15326173	4,6	+	-	+
At4g33050	EDA39	calmodulin-binding protein	15634699	3,4	+	+	+
At4g34390	XLG2	Extra-large GTP binding protein;	10394945	3,2	0	-	0
At4g35180	LHT7	LYS/HIS transporter 7	16607029	5,9	+	-	+
At4g37290		Unknown protein	NF	3,2	+	-	+
At5g13080	WRKY75	WRKY Transcription Factor	17322336	7,5	+	+	+
At5g26690		Heavy metal transport/detoxification protein	NF	7.0	N	-	N
At5g48540		33 kDa secretory protein-related	16115070	4,1	0	+	+
At5g54610	ANK	Ankyrin; SA induced	16307367	4,4	0	-	+
At5g58300		leucine-rich repeat transmembrane protein kinase	16280546	3,2	-	-	-
At5g58350	WNK4	WNK kinase	12506983	3.0	0	+	+
At5g64120		cell wall bound peroxidase; oxidative burst	16551688	5,7	+	+	+

With these parameters we identified 40 genes whose transcripts are increased in the mutant compared to the wild type. We evaluated our data to other microarray experiments and the senescence transcripts identified by Guo et al. (2004). In Figure 4 a VENN diagram illustrates the overlap between the *old9* genes and the different experiments.

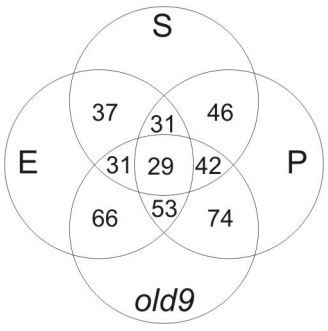

Figure 4. A Venn-diagram is used to visualize the overlap between the *old9* microarray dataset and that of other experiments. E: Microarray dataset of plants treated for 3 hours with ethylene; P: microarray dataset of plants exposed to *Phytophthora;* S: EST dataset of senescence associated transcripts.

The NASCARRAYS-32 was performed with seedlings treated for 3 hours of ethylene by F.F. Millenaar. The identified transcripts correlate for 36.8% with the *old9* transcriptome data. Next to that, 60% of the genes induced by ethylene in *old9* were previously identified during a plant defense experiment NASCARRAYS-123 by D. Scheel. Interestingly, the experiments of Millenaar and Scheel share an overlap of

65%, indicating that ethylene induces many plant defense associated transcripts. Importantly, 44.7% of the induced genes in *old9* have been implicated before in leaf senescence (Guo et al., 2004). Thus, as expected we find many ethylene-inducible genes but the notion that almost 50% of the genes are senescence-associated suggests early onset of leaf senescence in the mutant.

Ten transcripts are specifically up regulated in the *old9* mutant as compared to the other experiments. One of these encodes a flavonol 7-O-rhamnosyltransferase which is involved in the biosynthesis of flavonols that function as either cell protectant, signaling molecules or regulator of auxin transport (Yonekura-Sakakibara et al., 2007). Next to that the ABA synthesis gene *CTF2b* is up regulated in *old9* (Bilodeau et al., 1999). ABA was shown to antagonize ethylene induced cell death in maize and barley (Young and Gallie, 2000; Wang et al., 1999). The microarray analysis of ethylene treated plants further supports the notion that in *old9* cell wall associated transcripts are differentially expressed as compared to the wild type. In *old9* we observed up regulation of *ATVAMP275* (At2g32670) a SNARE-encoding gene involved in vesicle transport of membrane proteins as well as cell wall materials from the plasma membrane (Uemera et al., 2004). *PMR6* encodes a pectate-lyase gene which is 160-fold induced after ethylene treatment of the mutant and is involved in cell-wall modification (Vogel et al., 2002). The degradation products of the cell wall were shown to trigger a signaling cascade including a putative leucine-rich repeat transmembrane protein kinase (At5g58300) which is specifically induced in *old9* (Moscatiello et al., 2006). Another interesting gene that is up regulated in *old9* encodes a G-protein (At4g34390) which serves as a physical coupler between cell surface, 7 transmembrane (7TM) G-protein-coupled receptors (GPCRs) and downstream targets (Temple and Jones, 2007). Noteworthy is the up regulation of *IQD14* (At2g43680) which is involved in calcium-signaling. During nutrient starvation and senescence, autophagy is used to breakdown cellular components and regulate nutrient relocalization. The autophagy gene *ATG18* is 5 fold induced after ethylene treatment of *old9* and was shown before to function in nutrient remobilization during leaf senescence of *Arabidopsis* (Xiong et al., 2005). To determine the relation between *old9* transcripts identified during air growth and after ethylene treatment we performed a coexpression analysis (Figure 5). The analysis revealed that 15 of the genes identified after ethylene treatment correlate to the genetic networks identified in air-grown *old9* mutant plants. The BGL2 network covers 15 genes of which 9 after

ethylene treatment, and contains in total 10 identified SAGs (Guo et al., 2004). The second group that is built around TPS11 covers 16 genes of which 12 have been identified as SAG (Guo et al., 2004).

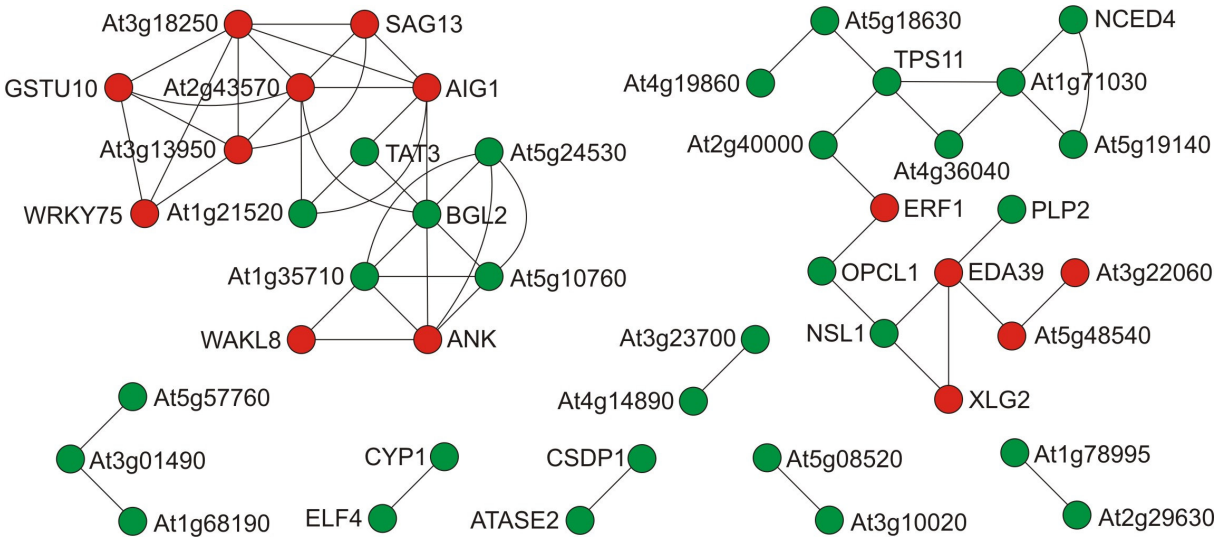

Figure 5. Correlation between transcripts of the microarray on air-grown *old9* and ethylene treated. Genes of the air-grown microarray dataset are represented by green spheres and those of the ethylene microarray dataset are shown in red. The relation between two is represented by lines. Lines are only drawn between genes with Pearson correlations >0.6. An S indicates that the gene is senescence associated.

Taken together this data shows that within 8 hours of ethylene treatment a senescence program is initiated in *old9*.

Cytokinin primes leaves for ethylene-induced senescence

Microarray analysis of air-grown *old9* mutants revealed that more than two third of the identified transcripts is responsive to cytokinin (Table 2). However, more than 2000 genes are responsive to cytokinin (Brenner et al., 2005) and only a small fraction was identified in the microarray. This suggest that only a specific set of cytokinin-responsive genes are important for the *old9* phenotype. Cytokinins are well-known for their ability to delay leaf senescence when applied exogenously (Lim et al., 2003) or when overproduced in transgenic plants (Gan and Amasino, 1995). However, the application or production of cytokinin in these experiments is always restricted to the last phase of leaf development just before or during the onset of leaf

senescence. The observation that long-term ethylene exposures of *old9*, starting at 8 days result in an early senescence phenotype (Chapter 2) suggests that the role of cytokinin in the *old9* phenotype is related to changes at early leaf development phase. Therefore we designed an experiment in which we tested the application of cytokinin at early and late developmental stages of the wild type and *old9*. If cytokinin promotes the onset of senescence by ethylene than application to the wild type should result in a phenocopy of the mutant.

Plants were given a cytokinin treatment at day 10, day 17 or at both days and grown in air till 21 days and subsequently treated for 3 days with ethylene. The visual senescence was assessed (Lohman et al., 1994) and presented in Figure 6 for wild type and *old9*.

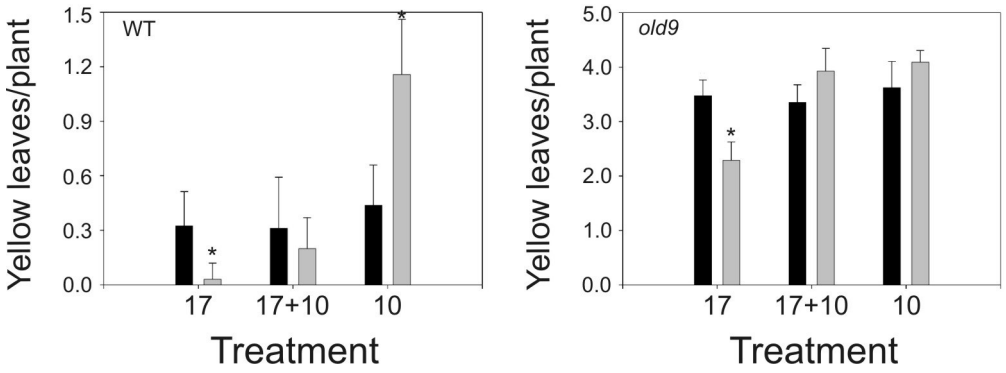

Figure 6. Cytokinin promotes the onset of ethylene-induced leaf senescence. Wild type and old9 plants were treated with cytokinin at day 17, day 10 or at both days as described in methods. After 21 days plants were treated with ethylene to induce senescence and 3 days later the number of yellow leaves per plant was scored. Experiments were repeated 3 times and similar results were obtained. Asterisks indicate statistical significance versus the mock treatment in each case ($P < 0.05$, Student's t test).

Application of cytokinin at 17 days resulted in a delay in the onset of senescence in both wild type and *old9* when compared to mock treatment, consistent with Lim et al. (2003) and Gan and Amasino (1995). Interestingly this effect disappeared when plants were treated at both 10 and 17 days for both wild type and *old9*. This suggests that early application of cytokinin results in either insensitivity to the application at day 17 or the application at early stage promotes the onset of senescence by ethylene which is again counteracted by application at day 17. Application at day 10 resulted in an increased senescence response of the wild type accession while this had no

effect on the senescence of the mutant. Taken together these results demonstrate that cytokinin can result in a partial phenocopy of the mutant phenotype in wild type when applied early in leaf development. More importantly, these results suggest that cytokinin can result in an ARC required for ethylene-inducible senescence when applied early in development.

Role of a hydroxyproline-rich glycoprotein in old9 response

The *old9* mutant plant has induced expression of several SAG genes when grown in air. The expression of these SAG genes might either prime *old9* for the induction of leaf senescence by ethylene or be a marker for this possible priming. Therefore we applied RNA interference (RNAi) to down-regulate one of these SAG genes and test whether this influences the induction of leaf senescence. RNAi constructs obtained from the Agrikola (Arabidopsis Genomic RNAi Knock-out Line Analysis) resource (Hilson et al., 2004) were screened and transformed to the mutant. This resulted in a stable RNAi line for the senescence associated hydroxyproline-rich glycoprotein (*Hrgp*) (At1g23040). Homozygous T2 plants of 3 independent lines were grown till 21 days and treated for 3 days with ethylene. The independent lines were obtained by transforming different copies of the same construct to different *old9* plants. Interestingly, all 3 lines showed a reduced senescence response compared to *old9* (Figure 7).

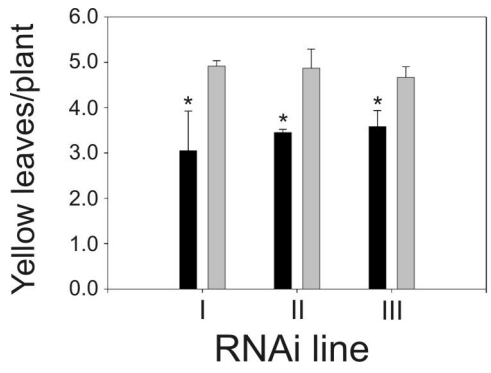

Figure 7. Visible yellowing of *old9* plants carrying a RNAi against HRGP. The number of yellow leaves of three independent RNAi lines is indicated and compared to *old9* control plants. The visible yellowing was scored and expressed as means ±SD of at least three replicates of 30 plants each. Plants that were grown for 24 d in air did not show any sign of senescence (not shown). Asterisks indicate statistical significance versus the *old9* mutant in each case (* $P < 0.05$, Student's t test). Black bars, RNAi line; Grey bars, *old9* mutant.

However, the senescence response is enhanced when compared to the wild type. These results demonstrate that *Hrgp* is an important determinant for the senescence

response of *old9* and supports the notion that at least one of the identified SAGs can prime a leaf for ethylene-inducible senescence.

Discussion

Plants are continuously interacting with the environment and adapt their growth and developmental strategy accordingly. During non-stress conditions the leaves of *Arabidopsis* undergo age-dependent senescence (Gan and Amasino, 1997; Quirino et al., 2000; Jing et al., 2002). The progression of senescence occurs in a coordinated manner starting from the tip of a leaf toward the base. The controlled breakdown of the leaf during senescence is in part to ensure effective remobilization of nutrients. Therefore it is not surprising that senescence is a highly regulated process involving many genetic programs (Buchanon-Wollaston et al., 2005; Keskitalo et al., 2005; van der Graaff et al., 2006). The two major plant hormones cytokinin and ethylene have well established opposite effects on leaf senescence. Here we report on the identification of an early ethylene-inducible senescence mutant that has increased expression of several SAG genes during air-grown conditions but no visual differences with the wild type. Interestingly, we demonstrate that cytokinin application at an early stage of plant development can promote the onset of leaf senescence by ethylene.

Endogenous levels of cytokinin drop concomitant with the progression of leaf development (Gan and Amasino, 1996) suggesting that the hormone plays a role during the development of the young leaf. The retardation of leaf senescence by cytokinin has been demonstrated by applying the hormone just before or during the onset of leaf senescence (Gan and Amasino, 1995; Lim et al., 2003). However, in our study we demonstrate that cytokinin can also have the opposite effect on the onset of leaf senescence. First the delay of senescence by applying cytokinin at 17 days is make ineffective when plants are also treated at 10 days with cytokinin. This might imply that application of cytokinin early in development has an opposite effect to application late in development. This is evident from the application at 10 days which is sufficient to induce early senescence after ethylene treatment in the wild type. Cytokinin is known to delay senescence by increasing the sink strength of the tissue (Balibrea Lara et al., 2004) and thus reverses the leaf senescence program which mobilizes nutrients from the senescing leaf to other parts of the plant. Thus by influencing the sink-to-source transition of a leaf, cytokinin might modulate the onset

of senescence. In support to this it was demonstrated in tobacco that application of cytokinin to leaf blades of decapitated plants is much less effective than application to intact plants to delay senescence (Singh et al., 1992) which was explained by suggesting that an increase of the sink strength only is not sufficient to delay senescence but also remobilization of nutrients to the tissue is required. Interestingly, detached leaves of *old9* senesce at the same time as wild type plants (Chapter 5), consistent with the idea that a sink tissue might be required for the accelerated senescence of *old9* leaves. Since we sprayed whole plants with cytokinin we interfered with the sink/source strength of all leaves. Therefore we propose that cytokinin promotes the onset of leaf senescence both in *old9* and treated wild type plants, by increasing the sink strength of young tissue. Endogenous cytokinin content and sensitivity to cytokinin regulates the developmental rate of *Arabidopsis* as mutants with a reduced cytokinin sensitivity had a slower rate of rosette leaf emergence and leaf expansion (Smalle et al., 2002), while plants with increased cytokinin sensitivity showed rapid chloroplast development and proliferation (Kubo and Kakimoto, 2000). Furthermore plants overproducing cytokinin develop more rosette leaves (Chaudhury et al., 1993). In *old9* several genes involved in different leaf developmental programs including cell elongation, vascular tissue formation and telomerase activity are down regulated compared to the wild type at 21 days, suggesting that the leaf developmental program is in a different phase than the wild type. Overexpression of the cytokinin receptor AHK3 or a downstream target ARR2 delays leaf senescence in *Arabidopsis* (Kim et al., 2006). Both transformants have a constitutive overexpression of cytokinin responsive genes. It would be interesting to determine the effect of ethylene on leaf senescence in relation to cytokinin with these mutants. This might reveal whether or not the *old9* phenotype is depending on a transient activation of cytokinin responsive genes or constitutive activation and if the subset of cytokinin responsive genes is important or all (Brenner et al., 2005).

The orderly visual appearance of cell death is in accordance with the induction of a senescence program in *old9*. Interestingly, the senescence starts at the tip of the leaf and thus the oldest leaf cells in *old9* (Ferreira et al., 1994) which is in agreement with the age-dependent progression of leaf senescence. The first leaf pair of 21 day *old9* mutants has increased expression of several SAGs which is not sufficient to induce early senescence under normal conditions but might prime *old9* for ethylene-induced

leaf senescence. Since SAG genes are associated with the last developmental phase they are a marker for an early ARC in the development of *old9* leaves. That these SAGs play a role in the ability of ethylene to induce early senescence is at least confirmed for one transcript. Transformation of the mutant with RNAi constructs directed against *HRGP* transcripts result in a decreased senescence response. Since the induction of leaf senescence by ethylene is age-dependent this suggests that reducing the expression of *HRGP* might interfere with the ageing of the leaf and thus causes a reduced response. HRGPs constitute one of the most abundant structural proteins in the plant cell wall and can confer resistance to pathogens in *Pearl millet* (Deepak et al., 2007). However, in *Arabidopsis* they have been implicated in embryo development (Hall and Cannon, 2002) but also in response to ABA (van Hengel and Roberts, 2003) suggesting that they play a role in growth and development of *Arabidopsis*. The accumulation of HRGP might represent a marker for a developmental change which is related to the progression towards leaf senescence.

The microarray analysis suggests important changes on the cell wall composition of *old9*. First of all a pectin synthesis gene is enhanced during air-growth, however during ethylene treatment a pectate lyase gene is induced 160 fold suggesting that the senescence of *old9* also involves a rapid degradation of the cell wall. Interestingly pectate lyase activity has been related to fruit ripening in strawberry and banana (Medina-Escobar, 1997; Asif and Nath, 2005) and resistance to Powdery Mildew when knocked-out (Vogel et al., 2002). Recently it has been demonstrated that pectic fragments of plant cell walls are able to induce defense and developmental responses (Moscatiello et al., 2006). Therefore the increased expression of the pectate lyase gene after ethylene treatment of *old9* might partially explain the abundance of plant defense genes after ethylene treatment. However, as shown, many plant defense genes are ethylene inducible and thus are probably not related to degradation of pectate. The effect of cell wall composition on the induction of leaf senescence has not been well described in literature and thus might be an aim for future research.

One question that remains is why *old9* does not show early leaf senescence during normal development in air. If the mutant is primed to senescence due to ARCs than it would be logical to conclude that this also affects the onset of developmental

senescence. A study by Jing et al. (2002) proposed the existence of a so-called senescence window. The senescence window is a developmental phase during which ethylene can induce premature leaf senescence. This model explains the absence of early developmental senescence in the mutant since OLD9 regulates at which developmental age a leaf can perceive ethylene to promote leaf senescence but not when to start to senesce. Since no early developmental senescence occurs this suggests that *old9* is not involved in the direct execution of senescence.

The conferred change by the *old9* mutation might be useful for controlling the ripening of crops or fruits. For example the simultaneous start of fruit ripening will make it possible to harvest all products at the same time.

Methods

Plant materials and growth conditions

Arabidopsis thaliana ecotype *Landsberg erecta* (Ler-0) was used in this study. The *old9* mutant was obtained from an EMS mutagenized collection (Jing et al., 2005). Plants were grown on either soil or half-strength Murashige and Skoog medium at 23℃ and 65% relative humidity with a day length of 16 h. The light intensity was set at 120 $\mu mol \cdot m^{-2} \cdot s^{-1}$. An organic-rich γ-ray radiated soil was used (Hortimea Groep, Elst, The Netherlands). Plants for ethylene exposure were treated in a flow-through chamber at 20 ℃ and a humidity of 40% under continuous illumination. The ethylene dosage was set at ~10 $\mu l\ l^{-1}$ as suggested by Chen and Bleecker (1995). Cotyledons or rosette leaves with over 5% yellow area of the leaf blade were judged as yellow as suggested by Lohman et al. (1994).

Tryphan blue staining

Appearance of cell dead were studied in whole leaf mounts stained with lactophenol-tryphan blue (10 ml of lacticacid, 10 ml of glycerol, 10 g of phenol, 10 mg of tryphan blue, dissolved in 10 ml of distilled water) (based on Keogh et al.,1980). Whole leaves were boiled for 1 min in the staining solution and then overnight decolorized in saturated solution of chloral hydrate. Subsequently the leaves were mounted in chloral hydrate and viewed under a light microscope.

RNA-isolation and RT-PCR

Total RNA was isolated using TRIZOL reagent (Sigma) according to the manufacturer's protocol. Five hundred nanograms of RNA were used as template for first-strand cDNA synthesis using 200U of RevertAid H-minus MMuLV reverse transcriptase (Fermentas, USA) and an oligo(dT21) primer. Primer pairs for real-time PCR were designed with open-source PCR primer design program PerlPrimer v1.1.10 (Marshall, 2004). The primer sequences are available upon request. Briefly, real-time PCR amplification was performed with 50 µL of reaction solution, containing 2 µL of 10-fold–diluted cDNA, 0.5 µl of a 10 mM stock of each primer, 1 µl of 25mM stock MgCl2 (Fermentas), 5 µl PCR buffer +Mg (Roche), 1 µl of a 1000x diluted SYBR-green stock (Sigma), 0.5 µl 100xBSA (New England Biolabs), and 1u of Roche Taq Polymerase. The PCR program was 2' at 94, 40x (94-10"/60-10"/72-25"), meltcurve. Obtained data was analyzed with BioRad software.

Microarray analysis

All the array experiments were carried out using the CATMA2 Arabidopsis GeneChip microarray containing 24576 gene-specific tags from *Arabidopsis thaliana* (http://www.catma.org). For each comparison, one technical replication with fluorochrome reversal was performed for each pool of RNA. cRNA were produced from 2 µg of total RNA from each sample using the Message Amp aRNA® kit (Ambion). Then 5 µg of cRNA was reverse transcribed in the presence of 300 units of SuperscriptTM II DNA polymerase (Invitrogen/Life technologies), cy3-dUTP and cy5-dUTP (PerkinElmer Life Sciences) for each slide. Slides were prehybridized for 1 h and hybridized overnight at 42℃ in 25% formamide. Slides were washed in 2× SSC and 0.1% SDS for 4 min, 1× SSC for 4 min, 0.2× SSC for 4 min, and 0.05× SSC for 1 min and dried by centrifugation. Raw data from each experiment were normalized in GeneSpring (Silicon Genetics, Redwood City, CA, USA) using the default procedure. GeneSpring was used to filter out genes detected in only one of the slides and to identify up- and down regulated genes for further analysis. The identified genes were compared to 2 experiments available from the Nottingham Arabidopsis Stock Centre (NASC) microarray database (http://affy.arabidopsis.info).

Coexpression analysis

Coexpression analyses were performed by using a Coexpression Gene Search algorithm on the RIKEN PRIMe web site. The Coexpression Gene Search program is a web-based application designed to identify correlated genes from gene expression data produced using Affymetrix Gene-Chip technology by the AtGenExpress consortium (RIKEN Plant Science Center and the Max-Planck Institute for Molecular Plant Physiology) deposited in TAIR. The database can be searched by using the AGI annotation. The ATTED-II release directly gives the correlation coefficients (Obayashi et al., 2007).

Cytokinin treatment

Plants were grown on soil for 21 days and subsequently exposed to ethylene for 3 days. During the growth of the plants a foliar spray technique was used to apply cytokinin. The synthetic cytokinin, benzyladenine (BA) was applied exogenously in a concentration of 50 µM dissolved in 0.5% Tween-20. As a control plants were treated by spraying with a solution of 0.5% Tween-20. Plants were sprayed in the morning and approximately 10 ml of solution was applied to a tray containing 104 plants.

RNAi lines

A 175-bp HRGP-specific fragment (clone CATMA1a22110) (from +308 to +482 of HRGP cDNA) was cloned into a binary vector by the AGRICOLA consortium to create a hairpin construct (Hilson et al., 2004). The pAGRIKOLA plasmid was verified according to the validation protocols by Hilson et al. 2004. The binary plasmid was introduced into *Agrobacterium tumefaciens* strain GV3101 together with the pSOUP helper plasmid through electroporation. *old9* mutants were subsequently transformed with the RNAI construct by the floral dip method (Clough and Bent, 1998).

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